# STUDIES ON EXPERIMENTAL HAPLOIDY IN SALAMANDER LARVAE

## I. Experiments with Eggs of the Newt, Triturus pyrrhogaster

CORNELIUS T. KAYLOR

*(From the Department of Anatomy, Medical College, Syracuse University and the Marine Biological Laboratory, Woods Hole, Massachusetts)*

### Introduction

The problem of experimental haploidy has been investigated mainly in the Amphibia, since these eggs can quite easily be induced to begin development with the reduced number of chromosomes and the larvae are convenient material for microscopical studies.

In general, the results of many experiments using different methods and species have been that the development of the haploid embryos is abnormal. If development continues into larval stages, the animals usually, at one time or another, show certain symptoms of haploidy, namely, stunted growth, reduced activity, and an edematous swelling of the body. This is in direct contrast to the fact that natural haploids are known to exist in many species of arthropods and that viable experimental haploid plants have been produced. However, some haploid salamander larvae have been experimentally produced which were relatively free of some of the serious symptoms associated with haploidy (see review of the literature by Fankhauser, 1937). The most striking example was a metamorphosed *Triton* larva obtained by Baltzer (1922) and Fankhauser. Even though some of the activities of this larva were subnormal, so far as its internal morphology was concerned (Fankhauser, 1938*a*), the effects of the haploidy were not too serious.

It would be safe to conclude from the observations of Fankhauser on this single *Triton* larva that the morphological and physiological abnormalities which have been common to the majority of haploids need further investigation. The present experiments were undertaken, therefore, with the purpose of obtaining a number of advanced haploid larvae for a further study of haploid morphogenesis, and of examining the possibilities of experimental treatment of some of the serious physiological symptoms of haploidy in these animals.

The observations to be presented in this paper describe the animals which were obtained and the attempts to reduce the edematous swelling

of the body which appeared during the development of most of these larvae. Microscopical studies on the haploid animals will be published later.

The experiments were performed at Syracuse and many of the observations were carried out at the Marine Biological Laboratory, Woods Hole. A preliminary report of these experiments has already been published (Kaylor, 1939).

## Material and Methods

Eggs of *Triturus pyrrhogaster* were selected for use, since, on the basis of Fankhauser's (1937) experiments on merogony with these eggs, it was believed that this species offered greater possibilities of extended haploid development than would others.

Eggs were obtained by anterior pituitary implantations. Development of the unoperated eggs was always normal. The female chromosomes were removed from the egg shortly after fertilization by means of a glass needle and small pipette (see puncturing method of Kaylor, 1937). All subsequent development then took place with only the male set of chromosomes. Control eggs, punctured to one side of the female nucleus, developed normally. These gave diploid embryos and larvae. Operated and control eggs were placed in a small dish of sterile modified Ringer solution,[1] and kept at a temperature of 20° to 22° C. until the animals had reached advanced larval stages (disappearance of yolk from the gut). From then on, the larvae were reared at room temperature varying between 22° and 26° C. Larvae were fed on *Enchytraeus*.

The corrosive sublimate, picric, acetic mixture of Michaelis was used for fixing yolk-laden embryos and larvae. Feeding larvae were fixed in Bouin's. In the sectioned material, Harris' hematoxylin was used for a nuclear stain and eosin or orange G in clove oil as a counter stain.

## Observations

### *General Results* (Table I)

Sixty-six out of 76, i.e., 87 per cent, of the punctured eggs of *Triturus pyrrhogaster* developed. The majority of these eggs were allowed to complete their developmental possibilities. Approximately 50 per cent of the developing eggs made at least an attempt at gastrulation, while 27 per cent developed beyond the gastrula stage. In a similar series of experiments with *Triturus viridescens* (Kaylor, 1937), only 10 per cent of all segmenting eggs developed beyond the gastrula stage.

[1] Solution according to Holtfreter except that the $NaHCO_3$ is omitted and the NaCl is reduced by one-half.

This comparison is of importance in experiments of this type where the main interest is in obtaining advanced stages of androgenetic development. It is also of interest to note from these observations that species differences are apparently an important factor in the extent and normality of haploid development, since in these two experiments the same methods were employed and the eggs were approximately the same size.

TABLE I

*Androgenetic development of eggs of Triturus pyrrhogaster*

| Stage Reached | |
|---|---|
| No cleavage | 10 |
| Early cleavage stages | 2 |
| Late blastula | 32 |
| Gastrula | 14 |
| Neural plate | 1 |
| Neural tube | 4 |
| Neural tube, eye vesicles | 1 |
| Gill buds, limb buds, heart beat | 7 |
| Larva (30 days), three finger buds | 1 |
| Larva (47 days), hind limb buds | 1 |
| Larva (60 days), hind limbs four toes | 1* |
| Larva (117 days), partly metamorphosed | 1† |
| Larva (120 days), beginning metamorphosis | 1‡ |
| Total | 76 |

* Killed by accident, not preserved.
† Died but preserved.
‡ Preserved while still developing.

### *Later Stages of Development*

*Evidence of Androgenetic Development.*—The main interest of the present experiments was the group of 12 larvae which developed to stages ranging from the limb bud to a larva 120 days of age.

Seven of these larvae were preserved when they became edematous and failed to develop beyond the limb bud stage. Of the remaining five, which were the oldest larvae obtained, two were preserved because certain of their abnormalities were not compatible with further development, one (the oldest) was fixed while still developing, one was killed by accident some time after the first direct test of its chromosome condition, and one died at metamorphosis.

The evidence for the androgenetic development of these larvae is summarized in Table II. The retardation of development during gastrulation and neurulation has been established in other experiments (Kaylor,

1937; Porter, 1939) as a reliable criterion for the successful removal of the egg chromosomes.

TABLE II *

Evidence for androgenetic development of advanced larvae.

| Stage of Fixation | Gastrulation and Neurulation | Pigment Cells | Chromosome Numbers |
|---|---|---|---|
| Limb bud, heart beat | Delayed | Small | — |
| Finger buds (30 days) | Delayed | Small | 12±1 |
| Hind limb buds (47 days) | Delayed | Small | 12±1 |
| Hind limbs (60 days) | Delayed | Large | 33+2–3 |
| Larva (117 days) | Delayed | Large | 29+; 33+2–3 |
| Larva (120 days) | Delayed | Small | 12±1; 24±2 |

* The chromosome numbers are the highest and the lowest in 20 or more counts made from tail fin epidermis and from various organs of the body, except in the case of the two triploids. In the 60-day triploid, there were three counts in all and only from the tail tip; in the 117-day animal, eleven counts from various regions of the body.

*Description of the Three Most Advanced Androgenetic Larvae.*— 1. The first larva, preserved on the 47th day (Fig. 1*b*), exhibited the typical symptoms of permanent haploidy: stunted growth, blunt head and small eyes, small gills, small pigment cells, sluggish reactions. During earlier development it had been edematous, which will be referred to in the discussion of edema, but at the time of fixation it was entirely

Figures 1 and 2, photographs of two of the oldest androgenetic larvae obtained: a haploid (Fig. 1), a haploid-diploid (Fig. 2). Figs. 3 and 4, photographs of two stages in the development of the haploid-diploid larva. Figs. 5, 6, 7, photographs of three stages in the development of the third advanced androgenetic larva: the triploid which died on the 117th day.

FIG. 1, haploid larva 47 days of age (*b*), diploid control of same age (*a*). Tail tip of both had been clipped. 4 ×.

FIG. 2, haploid-diploid larva 120 days of age (*b*), diploid control of same age (*a*). Tail tip of both had been clipped. *ca* 2 ×.

FIG. 3, the same haploid-diploid larva on the 21st day (*b*), diploid control of same age (*a*). Tail tip of *b* had been clipped. 4 ×.

FIG. 4, the same haploid-diploid larva on the 44th day (*c*), a diploid control about 3 days younger (*b*). Tail tip of both had been clipped. Diploid control (*a*) of same age as haploid-diploid larva. 2 ×.

FIG. 5, the triploid larva on the 21st day (*b*), a diploid control of same age (*a*). Tail tip of *b* had been clipped. 4 ×.

FIG. 6, the triploid larva on the 44th day (*c*), a diploid control about 3 days younger (*b*). Tail tip of both had been clipped. Diploid control (*a*) of same age as the triploid larva. 2 ×.

FIG. 7, the triploid larva on the 64th day (*b*), a diploid control of same age (*a*) with tail tip clipped. 2 ×.


FIGS. 1–7.

free of edematous swelling of the body which is so disastrous to most haploids. It was finally preserved, after it had exhausted the yolk supply in its gut, because its lower jaw was deformed and the animal was unable to take food. Introduction of macerated food through the mouth was unsuccessful.

All chromosome counts made so far in the tail fin epidermis and sections of the body have given the haploid number.

The existence of this larva demonstrates that advanced haploid larvae can be obtained with this method from whole eggs of this species. With larger numbers of animals it is not improbable that a greater number of advanced haploid larvae could be produced.

2. The second larva, fixed on the 120th day (Fig. 2*b*), unlike the majority of haploids, was not appreciably reduced in size in comparison to controls of the same age (Fig. 2*a*). Some of its morphological characteristics were, however, similar to those of other haploids, as, for example, the blunt nose and small head. In its reactions to stimuli the larva was as normal as any of the controls. This has not been a characteristic of all the other pure haploids obtained by a number of investigators. There had been an edematous condition of the belly and heart region during the limb bud stage, but this disappeared of its own accord and did not recur.

In spite of the fact that at the time of fixation this larva was unlike most haploids, there was the following evidence that it had developed with only the haploid male set of chromosomes: (1) A definite retardation in development at gastrulation and neurulation, which was evident in later stages (compare hind limb buds of this larva in Fig. 4*c* with a larva of the same age in Fig. 4*a* and with a control about 3 days younger in Fig. 4*b*). (2) Smaller pigment cells than those of controls (Figs. 3*b*, 4*c* compared to Figs. 3*a*, 4*a*, 4*b*). (3) The haploid chromosome number in ten nuclei of the tail tip epidermis of the young larva. Also the majority of epidermal nuclei (Fig. 8*a*) were smaller than diploid nuclei of controls (Fig. 8*b*).

However, when the entire animal was sectioned, preliminary chromosome counts in five different tissues showed that the larva was a mixture of haploid and diploid cells. Further investigation is needed to determine whether all organs are haploid-diploid. It seems probable, then, that regulation from haploidy to diploidy, which has been observed so frequently in parthenogenetically developed frog tadpoles (Parmenter, 1933, 1940; Porter, 1939; Kawamura, 1939), does occasionally occur in androgenesis in the newt.

Comparison of this larva (Fig. 2*b*) with the purely haploid larva (Fig. 1*b*) demonstrates, in part at least, the effect of haploidy-diploidy

upon the extent and normality of development. There is still the possibility, also, that a more normal morphological condition, which was independent of chromosome numbers, was obtained in this older larva.

3. A third larva died on the 117th day and was preserved. It had not completely metamorphosed. It was slightly larger than any of the controls of the same stage of development, possessed no apparent morphological irregularities, and was normally vigorous in all its reactions. No photograph was taken after the animal's death. However, in a photograph taken on the 64th day (Fig. 7*b*) it is seen that the larva appeared to be anatomically perfect and, even at this time, larger than controls of the same age (Fig. 7*a*). All larvae had been fed the same amount of food.

This larva was triploid, even though there was the suggestion that the animal had developed by means of androgenesis. The first prepara-

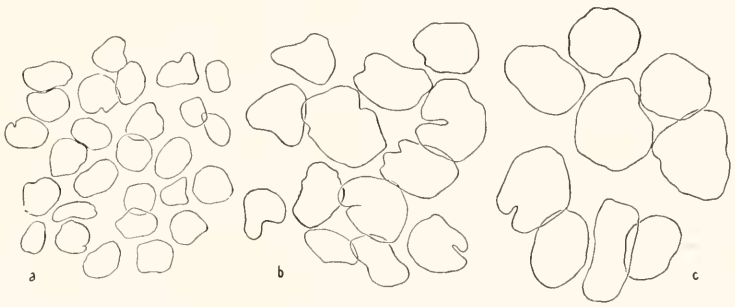


FIG. 8, camera lucida drawings of nuclei of epidermis cells of the haploid-diploid larva (*a*), of a diploid control (*b*), and of the triploid larva (*c*). 400 ×.

tion of tail fin epidermis offered 4 chromosome counts, all of which could be accepted as the triploid number. All nuclei in that piece of epidermis, a few of which are shown in Fig. 8*c*, were larger and less numerous than the diploid nuclei of controls (Fig. 8*b*). In a preliminary examination of sections of the entire larva, it was possible to make 7 chromosome counts from five different tissues (mesenchyme, cloacal epithelium, peritoneum, liver, heart). These counts were all approximately the triploid number, i.e., from $29+$ to $33+2-3$ (the triploid number being 36). It would appear, then, that this larva is entirely triploid and adds the eighth species to the list of Amphibia in which triploidy has been demonstrated.[2]

The evidence suggesting the origin of this triploid larva by means

[2] See review of literature by Fankhauser, 1938*b*; also see Kawamura, 1939, and Fankhauser, 1939.

of androgenesis is based on the following condition: a delay in development as compared to controls of the same age. The retardation of development, which was evident during gastrulation and neurulation, was still noticeable in the fore limb stage of the young triploid larva (Fig. 5*b* compared to Fig. 5*a*). Also the hind limb buds of this larva (Fig. 6*c*) were delayed in comparison to controls of the same age (Fig. 6*a*) and to controls about 3 days younger (Fig. 6*b*). Delayed development became less noticeable as the larva increased in age (Fig. 7*a* compared to Fig. 7*b*). The pigment cells were as large as or larger than those of the controls (as may be seen by magnifying Figs. 5*b*, 6*c* compared to Figs. 5*a*, 6*a*, 6*b*).


FIG. 9. Edematous haploid larvae (*a*, *b*, *c*, *d*, *e*, *f*, *g*) and a control (*h*) of the same age. Note the difference in size of the pigment cells of the haploid and diploid larvae. 4 ×.

There is no evidence as to the origin of triploidy in this case, but it must have occurred very early in development since only triploid chromosome numbers have so far been obtained in the entire larva.

*Experiments to Reduce Edema.*—The seven larvae which ceased development during the limb bud stage (Fig. 9, *a*, *b*, *c*, *d*, *e*, *f*, *g*) were edematous in the tissues of the heart and belly region. They were much smaller than controls (Fig. 9*h*) of the same age.

In two cases the edema began during the gill bud stage when pigment cells had appeared, the heart was beating, and Y-shaped blood islands were formed in the yolk region. In the other animals it appeared slightly later, at a time when circulation was established.

The development of the circulatory system of these larvae appeared to be abnormal, since in all cases there were localized accumulations of blood cells which persisted even after circulation had started in the rest of the body. This observation suggests that venous connection between the vitelline veins was not established, as Porter (1939) found in edematous frog larvae. The heart beat was normal. The pronephros

was probably established in all these larvae since the pronephric mound could be seen on the body surface. It is well established at this time in normal *Triturus pyrrhogaster* and venous connections of the cardinal veins are established in connection with the tubules. This suggests the functional activity of the tubules, since Armstrong (1932) has shown that in the fish, *Fundulus,* pronephric tubules become functional as soon as venous connections have developed in relation with the tubules.

As the edematous areas appeared they were opened under sterile conditions and the fluid allowed to escape. Then the animals were placed in sterile Clarke-Ringer solution.

In 6 cases, the edema returned within 12 to 24 hours in just as severe form as previously. The bloated areas were opened again and the larvae placed in sterile hypertonic salt solution but still the edema persisted. It gradually destroyed mesoderm and endoderm cells of the belly region and invaded the pericardial and head spaces. The heart action gradually became weaker until it was necessary to preserve the animals.

In the 7th larva (Fig. 9*a*), the edema returned in milder form and the animal lived for four days longer than the previous ones. However, its heart action gradually weakened and it was necessary to fix the animal. The head and fore limb region had differentiated slightly beyond the other six larvae.

The larva which was preserved on the 47th day (Fig. 1*a*) was also treated in the same manner for an edema which developed during the gill bud stage when circulation had been established. The condition disappeared completely and at the time of fixation it had not recurred.

A histological examination of all these larvae is in progress.

## Discussion

### *The Production of Advanced Larvae*

These results demonstrate that the majority of punctured eggs of *Triturus pyrrhogaster* made at least an attempt at development, and that a fairly high percentage of the segmenting eggs reached advanced stages of development.

It was also indicated that haploids of *Triturus pyrrhogaster* developed better than haploids of *Triturus viridescens.* Both species of eggs were practically the same size and were treated with identical methods. There are probably different factors in one species which determine the susceptibility of the developing eggs of that species to the effects of haploidy. Such variations between species (and genera) have been pointed out by other writers (P. Hertwig, 1923; Fankhauser, 1937; Porter, 1939).

However, the present experiments have produced no purely haploid

larvae which could equal the degree of differentiation obtained in the haploid *Triton* larva of Baltzer and Fankhauser. The reasons for this failure must await histological studies of the larvae which were obtained. There was the promising indication, on the other hand, that proper treatment of haploid larvae at critical periods in their development might possibly aid in lengthening the life of certain individuals.

One haploid-diploid larva was obtained which was apparently a normal individual since it developed to metamorphosis with no difficulty. It was pointed out that the success of extended development in this case may have been due to: (1) the normal number of chromosomes in many of its cells which may have affected physiological activities, and (2) a more normal morphological condition which occurred independent of the chromosome number.

Two triploid larvae were obtained in these experiments. All chromosome counts made so far in 5 different tissues of one larva have been triploid. The evidence for triploidy in the other larva is based solely on chromosome numbers and cell size in tail tip epidermis. The delay in development which was observed in both of these larvae might have been evidence of androgenesis. In this case, the triploid number of chromosomes must have originated in some manner from the sperm nucleus before first cleavage, because all chromosome numbers so far determined have been triploid. In order for this to have occurred it might be that the male haploid set of chromosomes divided once and then in some way one of these two sets divided again.

On the other hand, the delay in development might have been brought about by an entirely different set of circumstances: it may be that the female nucleus was left in the egg, retaining the second polar body (thereby giving a diploid female nucleus), and was then fertilized by the sperm nucleus. This would have produced a triploid embryo. It is impossible on the basis of the evidence at hand to say which of the two methods of origin of these triploid larvae was the most likely one.

### *Attempts to Reduce Edema*

All haploids of these experiments suffered from edema at varying stages of development. In the majority of cases it was impossible to reduce this condition by the use of various concentrations of salt solutions. In only one larva was the edema permanently reduced by this treatment; in this case it is possible that the success was due primarily to a concomitant regulation of certain internal structures, such as the circulatory and kidney systems. Preliminary studies on the sections of this larva have indicated that this may indeed have been the case. Suc-

cessful treatment by these methods in any haploid larva, then, would depend upon the chance that certain internal structures are normally and even hyperplastically developed.

One other larva, the haploid-diploid, was edematous at an early period. The condition disappeared voluntarily. It is possible that further histological study of this larva may give a clue to the factors concerned in its improvement.

Aside from the observations on the living animals that the circulation was abnormal, no adequate explanations for the persistence of edema in these larvae can be advanced until the histological examination has been completed.

## Summary

1. Eighty seven per cent of the punctured eggs of *Triturus pyrrhogaster* began development.

2. Advanced haploid larvae were obtained. Nine haploid larvae developed to stages ranging from the limb bud to a swimming larva 47 days of age. One haploid-diploid larva had begun to metamorphose at the time it was fixed.

3. Advanced androgenetic larvae can be obtained more readily in *Triturus pyrrhogaster* than in *Triturus viridescens: 27* per cent of the segmenting eggs developed beyond the gastrula stage in *Triturus pyrrhogaster* as compared to 10 per cent in *Triturus viridescens.*

4. Edema was the most common and serious abnormality associated with haploidy.

5. In the majority of cases it was impossible to reduce the edematous condition of the larvae with varying concentrations of salt solutions.

6. Two triploid larvae were obtained from punctured eggs. These larvae appeared to be entirely normal and slightly larger than the diploid larvae. This is the eighth species of amphibian in which triploidy has been observed.

## BIBLIOGRAPHY

Armstrong, P. B., 1932. The embryonic origin of function in the pronephros through differentiation and parenchyma-vascular association. *Am. Jour. Anat.,* **51**: 157–188.

Baltzer, F., 1922. Ueber die Herstellung und Aufzucht eines haploiden Triton taeniatus. *Verh. Schweiz. Natf. Ges., Bern,* **103**: 248–249.

Fankhauser, G., 1937. The production and development of haploid salamander larvae. *Jour. Hered.,* **28**: 2–15.

Fankhauser, G., 1938*a*. The microscopical anatomy of metamorphosis in a haploid salamander, Triton taeniatus Laur. *Jour. Morph.,* **62**: 393–413.

Fankhauser, G., 1938*b*. Triploidy in the newt, Triturus viridescens. *Proc. Am. Phil. Soc.,* **79**: 715–739.

Fankhauser, G., 1939. Polyploidy in the salamander, Eurycea bislineata. *Jour. Hered.,* **30**: 379–388.

HERTWIG, P., 1923. Bastardierungsversuche mit entkernten Amphibieneiern. *Roux' Arch.*, **100**: 41–60.

KAWAMURA, T., 1939. Artificial parthenogenesis in the frog. 1. Chromosome numbers and their relation to cleavage histories. *Jour. Sci. of the Hirosh. Univ., Ser. B, Div. 1,* **6**: 116–218.

KAYLOR, C. T., 1937. Experiments on androgenesis in the newt, Triturus viridescens. *Jour. Exper. Zool.*, **76**: 375–394.

KAYLOR, C. T., 1939. Experiments on the production of haploid salamander larvae. *Collecting Net,* **14** (No. 8): 169.

PARMENTER, C. L., 1933. Haploid, diploid, triploid, tetraploid chromosome numbers, etc. *Jour. Exper. Zool.*, **66**: 409–453.

PARMENTER, C. L., 1940. Chromosome numbers in Rana fusca parthenogenetically developed from eggs with known polar body and cleavage histories. *Jour. Morph.*, **66**: 241–260.

PORTER, K. R., 1939. Androgenetic development of the egg of Rana pipiens. *Biol. Bull.*, **77**: 233–257.